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PRINCIPAL INVESTIGATOR: Jonathan Chernoff, M.D., Ph.D.

CONTRACTING ORGANIZATION:
                            Institute for Cancer Research
                            Philadelphia, PA 19111

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Institute for Cancer Research
Philadelphia, Pennsylvania 19111
E-Mail: Jonathan.Chernoff@fccc.edu

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14. ABSTRACT

The NF2 product, Merlin, has recently been shown to inhibit p21-activated kinases (Paks), enzymes known to activate cell cycle progression and to induce changes in the actin cytoskeleton. These findings suggest that loss of Pak function might inhibit the abnormal growth and/or movement of cells lacking Merlin. We had proposed two aims: to test if loss of all Pak function affects signaling in NF2-/- cells and to test if Paks are required for tumorigenesis in an NF2 mouse model system. In the third year of this project, we published a manuscript describing the first selective small molecule inhibitor of group A Paks; completed crossing NF2 and Pak1-/- mice into a C3H genetic background; used a retroviral vector to express an enhanced Pak inhibitor (the Pak2 inhibitor domain) in normal and NF2 BBA (dominant negative) mutant mouse fibroblasts, and showed that loss of Pak function impedes NF2-driven cell proliferation and abnormal morphology; and used the same approach in xenograft experiments to show that loss of Pak function reduces NF2-induced tumor formation. With these advances, we have matched and in some cases exceeded our timetable for year three.

15. SUBJECT TERMS

NF2, GTPASE, Protein Kinase, Signal Transduction and Genetic Interactions

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Jonathan Chernoff, M.D., Ph.D.

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INTRODUCTION:

The goal of this project is to determine if group A p21-activated kinases (Paks) are important elements in signaling in neurofibromatosis type II (NF2). Our hypothesis is that inactivation of the *NF2* gene disrupts a signaling pathway emanating from the small GTPase Rac and its effector, p21-activated kinase (Pak). We propose that stimulation of the Rac/Pak signaling axis in cells lacking Merlin leads to changes in transcriptional activity and cytoskeletal dynamics, ultimately resulting in enhanced cell proliferation and motility, which are hallmarks of tumorigenesis. <u>If this hypothesis is correct, then inhibition of Pak signaling should disable the growth advantages of cells lacking Merlin.</u> We intend to test this theory using Pak loss-of-function cells and animals.

BODY: We set ourselves two specific tasks. These were:

Task 1. To determine if Pak function is required for mitogenic or morphogenic signaling in fibroblasts and Schwann cells lacking Merlin (Months 1-24):

      a.   Analyze the expression level and activity of group A Paks in fibroblasts and Schwann cells (Months 1-6).

      b.   Analyze effects of loss of Pak function on mitogenic and morphogenic signaling in mouse embryo fibroblasts lacking Merlin (Months 6-18).

      c.   Analyze effects of loss of Pak function on mitogenic and morphogenic signaling in mouse Schwann cells lacking Merlin (Months 18-24).

Task 2. To investigate the influence of Pak on the formation of tumors in transgenic mice expressing a dominant negative form of Merlin (SchΔ(39-121), by determining if crossing such mice with a) transgenic mice expressing a Pak inhibitor (PID), or b) *Pak1$^{-/-}$* mice affects their predisposition to the tumors typically seen in NF2 (Months 6-48):

      a.   Generate PID transgenic cells (Months 6-12).

      b.   Generate and analyze PID transgenic mice (Months 12-24)

      c.   Mate P0-SchΔ(39-121) mice with the PID transgenics and *Pak1$^{-/-}$* mice and analyze crosses (Months 24-48).

Progress

We have met, and in some cases, exceeded, our goals for the second year, as detailed below.

<u>Task 1</u>

    a) <u>Analyze the expression level and activity of group A Paks in fibroblasts and Schwann cells.</u> This was accomplished last year, as detailed in our previous progress report. The results showed that Pak1 is the predominant group A Pak expressed in both fibroblasts and Schwann cells but that Pak2 is also expressed at

reasonably high levels. These results confirmed our strategy to focus on these two isoforms of Pak in our genetic and biochemical experiments.

b) <u>Analyze effects of loss of Pak function on mitogenic and morphogenic signaling in mouse embryo fibroblasts lacking Merlin.</u> As described in our previous progress report, we created both retroviral and adenoviral expression vectors encoding the Pak1 inhibitor domain (PID). These had to be altered slightly for the following reason. We had earlier reported (1) that expression of a control, inactive version of the PID (PID L107F), unexpectedly slows cell cycle progression. I have subsequently learned from Ed Manser that the Pak1 PID, as well as the mutant form, PID L107F, binds to the Fragile X (FGX) protein (personal communication). This property makes it difficult to interpret results obtained with this reagent. Fortunately, there is an easy fix. The Pak2 and Pak3 PID do not bind FGX, yet retain full ability to suppress Pak activity (data not shown). Therefore, we are reconfiguring our retroviral vectors to encode the Pak2 PID and its inactive control, Pak2 L106F (Fig. 1).

In addition, as the result of a related research project, we developed a small molecule inhibitor of Pak, termed IPA3. This compound inhibits Pak1 at a K_i of 2.4 μM in fibroblasts (Fig. 2), and is also a potent inhibitor of the other two group A Paks, Pak2 and Pak3. IPA3 does not inhibit the three group B Paks at 10 μM concentrations. Remarkably, when tested against a panel of ~240 protein kinases, we found that the specificity of IPA3 is comparable to that of some of the best protein kinase inhibitors known, such as the Abl inhibitor Imatinib (Gleevac) and the Rho kinase inhibitor Y-27632 (Table 1). IPA3 thus represents an additional reagent for testing the role of group A Pak function in NF2-deficient cells.

c) As planned, we have not initiated studying the effects of PID expression in Schwann cells until we gather more data from the fibroblast system, because Schwann cells are relatively difficult to isolate and manipulate.

<u>Task 2</u>

a) As described in the previous progress report, we altered the order of experiments to reflect certain logistical realities; namely, that we already had available $Pak1^{-/-}$ and $Pak2^{flox/flox}$ mice, whereas we are still in the process of generating transgenic mice expressing the PID. For these reasons we focused our initial efforts on task 2c: to cross mice transgenic expressing dominant negative NF2 in Schwann cells (P0-SchΔ(39-121))(2) with $Pak1^{-/-}$ mice. We have nearly completed one version of this experiment. We studied two groups of approximately 30 mice each: P0-SchΔ(39-121) $Pak1^{+/+}$ and P0-SchΔ(39-121) $Pak1^{-/-}$. Over a one-year period, we watched these cohorts for the development of Schwannomas and other malignancies. Although the experiment is ongoing, the initial data look very encouraging. That is; we found that 5/29 P0-SchΔ(39-121) $Pak1^{+/+}$ mice developed NF2-related pathologies (schwannomatosis, nerve sheath tumors, sarcomas), whereas 1/33 P0-SchΔ(39-121) $Pak1^{-/-}$ mice developed NF2-related pathologies. These preliminary data suggest that our main hypothesis is correct: Pak1 is required for the efficient development of NF2-related pathologies, and

also support the corollary idea that Pak inhibitors might be useful in the treatment of NF2.

During the course of this experiment, we learned that the mixed C57 Bl6/C129 background is not ideal for studying the effects of the NF2 transgene (P0-SchΔ(39-121). This is because disease is slow to develop in this mixed strain background, making the experiments difficult to complete in a reasonable timeframe. The creator of the P0-SchΔ(39-121) transgenic mice, Marco Giovannini, has found that the C3H background is much more favorable for analysis (M.G., personal communication), as schwannomatosis develops in all the transgenic mice by three months of age. We have therefore started to cross P0-SchΔ(39-121) mice and *Pak1*$^{-/-}$ mice into the C3H background (four crosses so far), in anticipation of repeating the studies listed above. We expect the complete results to be available in about 1.5 years.

b) As reported previously, we added a new task to those originally specified in the grant proposal. We have transduced a dominant negative form of NF2 (the NF2 BBA mutant (3)) into MEFs derived from C57/Bl6 wild-type, *Pak1*$^{-/-}$, or *Pak2*$^{-/-}$ mice, and are currently characterizing these for growth properties. These experiments are a few months behind schedule because the NF2 BBA mutant was temporarily unavailable from AddGene and from the Jacks lab at MIT. We have now made the mutant ourselves. Our plan is to inject the NF2-transduced cells into nude mice and test for tumorigenesis. It has been shown previously that mouse 3T3 fibroblasts transduced with NF2 BBA are tumorigenic when injected into the flanks of nude mice, resulting in palpable tumors within 1 week of injection (3). Therefore, we can test if loss of *Pak1* or *Pak2* in MEFs affects the ability of cells expressing NF2 BBA to induces tumors in this xenograft model. These experiments should be complete within twelve months, and will give us a rapid assessment about the role of Paks in NF2 tumorigenesis.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

1. Ascertained expression levels of endogenous group A Paks in MEFs and Schwann cells.
2. Constructed and tested viral expression vectors for the Pak1 PID.
3. Identified and tested a small molecule inhibitor of group A Paks.
4. Obtained preliminary results from crosses between NF2 with *Pak1*$^{-/-}$ mice.
5. Began crossing NF2 and *Pak1*$^{-/-}$ mice into C3H genetic background.
6. Created dominant negative NF2 BBA mutant and subcloned it into retroviral vector, and used the resulting virus to infect *Pak1*$^{-/-}$ and *Pak2*$^{-/-}$ MEFs in preparation for xenograft tumorigenesis studies.

REPORTABLE OUTCOMES:

Applied for and obtained a grant from the Children's Tumor Foundation, Drug Discovery Initiative Award #2007B-05-003 "Synthetic Lethal Screen of FDA-approved Drugs in NF2".
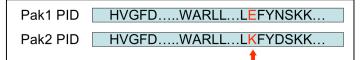
CONCLUSION:

We have engaged in the initial steps of this project in line with our overall plans. There are two new approaches that we have added: i) the use of a small molecule Pak inhibitor to supplement our cell-based studies and ii) xenograft studies as a quick readout of Pak's role in NF2-related tumorigenesis.
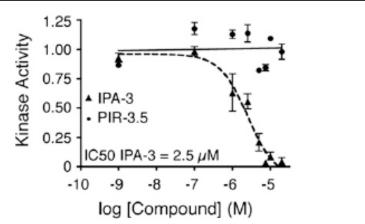
REFERENCES:

1. Thullberg, M., Gad, A., Beeser, A., Chernoff, J., and Stromblad, S., The kinase-inhibitory domain of p21-activated kinase 1 (PAK1) inhibits cell cycle progression independent of PAK1 kinase activity. *Oncogene*, **26**:1820-8, 2007.
2. Giovannini, M., Robanus-Maandag, E., Niwa-Kawakita, M., van der Valk, M., Woodruff, J.M., Goutebroze, L., Merel, P., Berns, A., and Thomas, G., Schwann cell hyperplasia and tumors in transgenic mice expressing a naturally occurring mutant NF2 protein. *Genes Dev.*, **13**:978-986, 1999.
3. Johnson, K.C., Kissil, J.L., Fry, J.L., and Jacks, T., Cellular transformation by a FERM domain mutant of the Nf2 tumor suppressor gene. *Oncogene.*, **21**:5990-7., 2002.
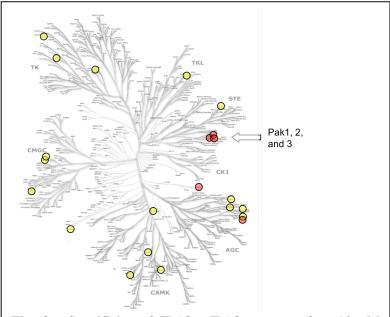
APPENDIX:

Figs 1 - 3

Fig. 1. New Pak Inhibitor Domain (PID) Construct. During the course of these studies, we found that the PID from Pak1, but not the PID from Pak2, binds to the Fragile X (FGX) protein. This feature complicates the use of the Pak1 PID as an inhibitor of endogenous Paks. The Pak2 PID bears a lysine residue in place of a glutamic acid (red arrow), and does not bind FGX.

Fig. 2. Potency of Pak inhibitor IPA3. IPA-3 inhibits Pak1 kinase activity with low micromolar potency. Pak1 was preincubated with the indicated concentrations of IPA-3 or PIR-3.5. Kinase reactions were started by addition of Cdc42, MBP, and a mixture of 1 mM ATP and [g-32P]ATP. Kinase activity is reported as phosphate incorporation into MBP expressed as a ratio to MBP phosphorylated in reactions in the presence of solvent alone (1% DMSO). Data are represented as the mean ± SEM.

Fig. 3. Specificity of IPA3. IPA3 was tested at 10 uM against a panel of 240 protein kinases. Red spheres represent >90% inhibition; yellow spheres >40% inhibition.